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**Abstract:** The ability to adjust skin darkness to the background is a common phenomenon in fish. The hormone  $\alpha$ -melanophore stimulating hormone ( $\alpha$ MSH) enhances skin darkening. In Mozambique tilapia, *Oreochromis mossambicus* L.,  $\alpha$ MSH acts as a corticotropic hormone during adaptation to water with a low pH, in addition to its role in skin colouration. In the current study, we investigated the responses of this fish to these two environmental challenges when it is exposed to both simultaneously. The skin darkening of tilapia on a black background and the lightening on grey and white backgrounds is compromised in water with a low pH, indicating that the two vastly different processes both rely on  $\alpha$ MSH - regulatory mechanisms. If the water is acidified after 25 days of undisturbed background adaptation, fish showed a transient

pigmentation change but recovered after two days and continued the adaptation of their skin darkness to match the background. Black backgrounds are experienced by tilapia as more stressful than grey or white backgrounds both in neutral and in low pH water. A decrease of water pH from 7.8 to 4.5 applied over a two-day period was not experienced as stressful when combined with background adaptation, based on unchanged plasma pH and plasma  $\alpha$ MSH and Na levels. However, when water pH was lowered after 25 days of undisturbed background adaptation, particularly  $\alpha$ MSH levels increased chronically. In these fish, plasma pH and Na levels had decreased, indicating a reduced capacity to maintain ion-homeostasis, implicating that the fish indeed experience stress. We conclude that simultaneous exposure to these two types of stressor has a lower impact on the physiology of tilapia than subsequent exposure to the stressors.

General and Comparative Endocrinology  
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Dear Dr. Does,

Please find included our manuscript entitled "Background adaptation and water acidification affect pigmentation and stress physiology of tilapia, *Oreochromis mossambicus*", which we hope you will consider for publication in GCE. The work described in this paper concerns the responses of tilapia to backgrounds and/or water acidification, focusing on pigmentation responses and on physiological reactions. We confirm that the work is original and will not be submitted for publication elsewhere until a decision has been made as to its acceptability for publication in GCE.

Kind regards,

On behalf of all authors,

Gert Flik

**Background adaptation and water acidification affect pigmentation and stress physiology of tilapia, *Oreochromis mossambicus***

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## Abstract

The ability to adjust skin darkness to the background is a common phenomenon in fish. The hormone  $\alpha$ -melanophore stimulating hormone ( $\alpha$ MSH) enhances skin darkening. In Mozambique tilapia, *Oreochromis mossambicus* L.,  $\alpha$ MSH acts as a corticotropic hormone during adaptation to water with a low pH, in addition to its role in skin colouration. In the current study, we investigated the responses of this fish to these two environmental challenges when it is exposed to both simultaneously. The skin darkening of tilapia on a black background and the lightening on grey and white backgrounds is compromised in water with a low pH, indicating that the two vastly different processes both rely on  $\alpha$ MSH - regulatory mechanisms. If the water is acidified after 25 days of undisturbed background adaptation, fish showed a transient pigmentation change but recovered after two days and continued the adaptation of their skin darkness to match the background. Black backgrounds are experienced by tilapia as more stressful than grey or white backgrounds both in neutral and in low pH water. A decrease of water pH from 7.8 to 4.5 applied over a two-day period was not experienced as stressful when combined with background adaptation, based on unchanged plasma pH and plasma  $\alpha$ MSH and Na levels. However, when water pH was lowered after 25 days of undisturbed background adaptation, particularly  $\alpha$ MSH levels increased chronically. In these fish, plasma pH and Na levels had decreased, indicating a reduced capacity to maintain ion-homeostasis, implicating that the fish indeed experience stress. We conclude that simultaneous exposure to these two types of stressor has a lower impact on the physiology of tilapia than subsequent exposure to the stressors.

## Introduction

Many poikilothermic animals have the ability to adjust the hue (observable colour; Trujillo et al., 1996) of their skin to the colour of their background by a combination of swift (physiological) and longer term (morphological) pigmentation changes (Bagnara and Hadley, 1973). Pigment cells, or chromatophores, can undergo changes in cell number, cell size, pigment content, pigment dispersion within the cell, cell migration and any combination of the former. A change in hue that involves a shift from light to dark or *vice versa* results from alterations in the state of black pigment cells, melanophores. During morphological pigmentation changes, numerical density and pigment content of melanophores can be decreased or increased upon adaptation to a white or black background, respectively. Physiological pigmentation changes comprise a relocation of the melanin granules within the cell, enabling a fast regulatory mechanism. In both mechanisms of pigmentation change, the same hormones are involved (Fujii, 2000).

$\alpha$ -Melanophore-stimulating-hormone ( $\alpha$ MSH) is classically known for its ability to induce pigment dispersion within pigment cells (Bagnara and Hadley, 1973), and is also involved in the regulation of melanin synthesis in mammals and fish (Eys and Peters, 1981; Halaban, 2000). Next to these pigment regulatory functions, in fish  $\alpha$ MSH serves as a satiation signal in feeding behaviour (Cerdeira-Reverte et al., 2003) and acts as corticotrope in the chronic phase of the stress response (Wendelaar Bonga, 1997). Plasma  $\alpha$ MSH levels increase after temperature shock (Sumpter et al., 1985), during confinement combined with air exposure (van der Salm et al., submitted) and during chronic exposure to acidified water (Lamers et al., 1991).

In addition to adaptation to a background, changes in hue can also be part of communication signals (Hulscher-Emeis, 1992; Lamers et al., 1991; Moyle and Cech Jr., 2000). In Mozambique tilapia (*Oreochromis mossambicus* L.), as in many other cichlid species, social status is reflected by the skin colour pattern of the fish, with the dominant male showing a much darker hue than submissive conspecifics. The ability of tilapia to alter the pigmentation pattern of their skin both very quickly and over a longer period of time makes them a very suitable model for background adaptation studies (Eys and Peters, 1981).

Furthermore, during adaptation of tilapia to low water pH,  $\alpha$ MSH-cell activity and plasma levels were significantly higher than in fish kept in water of pH 7.4 (Lamers et al., 1992). Other studies by the same authors indicated  $\alpha$ MSH to have corticotropic effects by

stimulating directly the release of cortisol from the interrenal tissue in the head kidney (Balm et al., 1995; Lamers et al., 1992).

These findings point to roles for  $\alpha$ MSH in tilapia in both pigmentation control and in the stress response. To further identify this role of  $\alpha$ MSH in these processes, we studied the interaction between background adaptation and the response to chronic low water pH. We assessed physiological responses (plasma hormones, glucose, lactate, Na and K) and determined the pigmentation response by analysis of scales taken from a defined location and assessing the area of the scale covered by melanophores as a parameter for the darkness of the body.

## **Materials and methods**

### *Animals*

Male and female tilapia, weighing  $66 \pm 17$  gram (means  $\pm$  sd; n=230) were obtained from laboratory stock, kept in large full-glass tanks. Following a time 0 control sampling (12 fish), fish were distributed over 15 experimental tanks containing 50L tap water of pH 7.8. Water temperature was 24° C and fish were kept at a day/night rhythm of 12 L:12 D. Fish were daily fed commercial tilapia food (Tilapia 3.0, Trouw, Putten, The Netherlands).

### *Experimental set up*

The walls of each experimental tank were covered with self-adhesive black (black background; B) or white foil (white background; W). The control tanks (control full-glass, grey background; G) were fitted with light-permeable one-sided see-through foil. In this way, disturbance of the fish by external movements or other stimuli was kept at a minimum and was similar for all groups, and the fish kept on the full-glass, grey underground served as extra controls, comparable to the background situation experienced in the rearing tanks (Table 1).

A first group of fish was transferred from the stock tanks to the experimental tanks and was allowed to adapt to the different backgrounds for 25 days. Experimental tanks were sampled at day 2, 8 and 25. This group is identified as the N (neutral water) treated group. A second group of fish was transferred and in the first two days after the start of the experiment the pH of the water was decreased from 7.8 to 4.5 by addition of H<sub>2</sub>SO<sub>4</sub> (Lamers et al., 1994). These fish were left to adapt to both the different background colours and the low water pH



for 25 days and tanks were sampled at day 2, 8 and 25. This group of fish is referred to as the pH group. The third group of fish was left to adapt undisturbed in neutral water to the different backgrounds for 25 days, before the pH of the water was lowered gradually over a two-days period (day 25 to 27) from pH 7.8 to pH 4.5 and kept at this pH for another 23 days (day 27 to day 50). These experimental tanks were sampled at day 27 (immediately after the pH drop) and at days 33 and 50. This group is referred to as the NpH (neutral followed by low pH water) treated group (see Table 1).

Throughout the experiment, pH of the water was monitored daily (Radiometer PHM, Copenhagen, Denmark; pH ranging between 7.4 and 7.9 for neutral water and 4.2 and 4.8 for acidified water) and adjusted if required.

### *Sampling*

Upon sampling, all fish were taken from a tank in a single scoop and euthanized in 0.2 % 2-phenoxyethanol. Blood was drawn from the caudal vessels, with syringes containing 35 µl of 2 % Na-EDTA to prevent clotting, and transferred to Eppendorfs containing 1 TIU of aprotinin to prevent proteolysis. The blood was spun at 4 °C for 10 minutes at 13,500 rpm, after which the supernatant plasma was transferred to fresh Eppendorf vials and quickly frozen.

After blood sampling, whole body pictures were taken of each fish. Then a scale was taken from each fish from the third row of scales below the lateral line on the left-hand side of the fish, approximately the sixth scale to the right of the operculum. Scales were fixed in 35% formalin and photographed later under a Leica Stereoscope (MZFLIII) with a Leica DC 500 digital camera. Fixation of the scales did not macroscopically affect melanophore features.

The pituitary gland was dissected and immediately frozen until further analysis.

### *Light microscopic analysis of the scales*

The digital photographs of the scales were analysed for the percentage of the scale covered by the melanin of melanophores. This method does not include the dispersion state or the number of individual melanophores but gives an indication of the lightness or darkness of the scale and therewith of the hue of the body. We used Adobe Photoshop 7.0 to measure the area (pixels) of the scales covered by epidermis and then measured the area (pixels) covered by melanin using the “Magic Wand” selection tool. By dividing the two values, a melanophore coverage expressed as a percentage of the scale (epidermis) was obtained.

### *Physiological parameters*

Pituitary glands were homogenized mechanically on ice in 100  $\mu$ l of 0.1M HCl. After centrifugation (13,600 rpm, 4° C) for removing membranes and cellular debris, the supernatant was used to assess the total amount of  $\alpha$ MSH in the pituitary glands. The  $\alpha$ MSH concentration in the plasma and in the pituitary gland was determined as described by Arends et al. (1999). The antiserum used for the  $\alpha$ MSH radio immunoassay cross-reacts for 100% with des-, mono- and di-acetyl  $\alpha$ MSH (Vaudry et al., 1978), and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum albumin (Zoest et al., 1989). The detection limit was 25.2 pg/ml sample. To determine cortisol concentrations, a RIA was used as described in detail by Metz et al. (2003). Radioactivity was quantified using a Cobra II  $\gamma$ -counter (Packard Instruments). Plasma glucose, ions (Na, Cl) and pH were measured with the Stat Profile® pHox® Plus L Analyser (Nova Biomedical) on the same plasma.

### *Statistical analysis*

Differences between groups were tested for significance with a two-way analysis of variance (ANOVA), followed by Dunnet's C post hoc tests when significant differences were found. The percentage of scale coverage by melanophores was arcsine transformed (Sokal and Rohlf, 1995) and then tested with the same methods. All values are expressed as means  $\pm$  sd. Statistical analyses were performed using SPSS 12.0.1 statistical software.

## **Results**

### *Pigmentation*

Two days after transfer to the experimental tanks, scales of fish in neutral water (N) were temporarily lighter on all backgrounds compared to the initial control fish sampled at time 0 (Figure 1A). This paling response was statistically significant only for fish from grey (NG) and white (NW) backgrounds. There was no difference in scale pigmentation between fish just after the pH drop during the first two days (pH group) and fish kept in neutral water for that time. While pHG and black background (pHB) fish subsequently turned darker again, pHW fish kept turning lighter throughout the experiment. pHG fish showed lower melanophore coverage values than NG fish, a difference that was significant at day 8

( $P < 0.05$ ). On the other hand, pHW fish showed slightly higher melanophore coverage values compared to NW fish (not significant). NG and NB fish had significantly higher coverage values than NW fish ( $P < 0.05$ ) and NB fish were visibly darker (Figure 1B).

The pH decline after 25 days of undisturbed adaptation evoked a similar response: NpHB fish showed a transient paling response, while NpHW fish on the other hand showed a slight transient darkening of the body. In the 23 days following this pH drop, NpHG and NpHW fish continued to further pale, a process that had started during the first 25 days of the experiment. NpHB fish became darker during this period. The correlation between the darkness of the background and the scale darkness was highly significant ( $P < 0.001$ ,  $r = 0.551$ ; Table 3).

### *Plasma parameters*

Plasma pH was not significantly influenced by the gradual acidification of the water in any of the experimental groups (Table 2). Values varied between 7.1 and 7.4 for all fish and showed a trend, on black and white backgrounds, to be lower in the pH fish. Values were generally lower in NpH fish compared to the pH fish, on all backgrounds.

Plasma cortisol levels were not affected by the pH decrease during the first two days after transfer to the experimental tanks (Figure 2A). Only in NG fish a significant increase in cortisol levels was observed (day 2; Figure 2A). For these fish, the levels of cortisol remained higher in neutral water fish until day 25, yet this difference was not significant. pHB and pHW fish showed an increase up to day 8, a rise which was absent in neutral water fish. NpH fish showed an increase in plasma cortisol levels upon a two-day decrease in water pH on all backgrounds. This increase was not significant compared to the day 25 values. These fish showed a high individual variation in cortisol values. However, in NpHB fish cortisol levels still increased up to day 33, whereas in NpHW fish a decrease is visible at this time point. These values differed significantly from each other ( $P < 0.05$ ).

Plasma  $\alpha$ MSH concentrations were not significantly different between fish from different backgrounds throughout the experiment (Figure 2B). Interestingly,  $\alpha$ MSH levels did not differ between fish from low pH water and fish from neutral water during the first 25 days of the experiment, but when the pH was lowered after 25 days of undisturbed adaptation in the NpH fish an increase could be seen of  $\alpha$ MSH concentrations on all backgrounds, significant in the NpHG fish only. In these fish,  $\alpha$ MSH levels declined afterwards, while levels remained elevated in NpHB and NpHW fish.

The total amount of  $\alpha$ MSH present in the pituitary glands is shown in Figure 2C. Increases were seen in the first two days after the start of the experiment in both the pH fish and in the NpH fish on all backgrounds, indicating that the pituitary  $\alpha$ MSH synthetic machinery was up-regulated. Levels returned to control values after 2 to 8 days. The total  $\alpha$ MSH content of the pituitary gland was not affected by background or by water pH, applied either separately or combined.

Plasma glucose concentrations (Table 2) showed no significant differences between backgrounds. Also, water pH did not significantly influence the glucose levels. Lactate levels (Table 2) showed a decline throughout the experiment on all backgrounds, which was not significantly altered by water pH. Plasma Na levels showed an increase during the first 25 days of the experiment in both the low pH and the neutral water fish (Table 2). In pHB and pHW fish higher Na levels were found than in neutral water fish. NpHG and NpHW fish showed a steep drop in plasma Na concentrations. This decrease was significant in NpHW fish. Plasma K levels were not influenced by background or by water pH (data not shown).

Correlation studies combining all data indicate that overall, plasma  $\alpha$ MSH and glucose levels are positively correlated with decreasing water pH, whereas plasma cortisol levels and the scale melanophore coverage values are positively correlated with the darkness of the background (Table 3). Fish with a higher  $\alpha$ MSH pituitary content show higher plasma cortisol levels, and lower plasma  $\alpha$ MSH concentrations. Plasma Na levels are negatively correlated with low water pH and plasma  $\alpha$ MSH levels, but are not affected by plasma cortisol concentrations (Table 3).

## **Discussion**

The major conclusions drawn from this study are three-fold. First, a black background appears to be experienced as more stressful than grey or white backgrounds. Second, in acidified water a black background is experienced as an extra stressor (the low water pH itself is a stressor as well). Third, and surprisingly, when the water is acidified following background adaptation the stress response is exaggerated compared to the simultaneous application. Both white and black backgrounds further exaggerate the response to low water pH.

*Pigmentation – Effects of background adaptation*

Tilapia from a black background were consistently darker than fish from white and grey backgrounds as judged by the scale melanophore coverage values. For the latter groups these scale coverage values were similar. Morphological background adaptation, a long term process, is not so much a result of melanosome reallocation within the cell but rather a result of either degeneration of melanophores on a light background or the formation of new melanophores on a dark background (Sugimoto, 1993; 2000; 2002). The procedure to score the scale coverage by melanophores does not discriminate between scale coverage changes either as a result of changes in melanin dispersion state of individual melanophores, or of changes in cell numbers. However, previous research on Mozambique tilapia has shown that long term background adaptation is indeed a result of changes in the numerical density of melanophores (Eys and Peters, 1981).

The adaptation to a light background involves degeneration of melanophores. According to (Sugimoto et al., 2000) apoptosis of melanophores is induced primarily by sympathetic signals (presumably norepinephrine) to the melanophores. A role was also hypothesised for melanin-concentrating hormone (MCH; Baker, 1993; Sugimoto, 2002), another hormone that affects melanosome motility.

The process of dark background adaptation is under the influence of both peptide hormones (such as  $\alpha$ MSH) and catecholamines. The increase in melanophore numbers during dark background adaptation is thought to be under control of  $\alpha$ MSH. This hormone can stimulate melanogenesis on the longer term by up-regulation of the expression of melanogenic genes (Sugimoto, 2002). However, the presence of many other factors such as growth factors, keratinocytes, fibroblasts and endothelins, is essential for this process to occur (Sugimoto, 2002). In the present study, plasma  $\alpha$ MSH concentrations were not different between fish kept on a grey, a white or a black background, although the darkness of the skin clearly differed between these groups. These results are in line with studies on other fish, in which no correlation was found between the hue of the body and plasma  $\alpha$ MSH levels. This may relate to changes in isoform frequency: the acetylation degree ( $\alpha$ MSH occurs as des-, mono- and di-acetylated isoforms) is an important determining factor in the bioactivity of the hormone. For instance, di-acetyl  $\alpha$ MSH is the most potent corticotrope in tilapia (Lamers et al., 1992).

The involvement of  $\alpha$ MSH in pigmentation in fish has been reported for several species, such as catfish, eel and trout (Baker et al., 1984), while in arctic charr, flounder, gilthead sea bream and red porgy such an involvement could not be demonstrated (Arends et al., 2000; Baker et al., 1984; Höglund et al., 2002; Salm et al., 2004). Earlier conclusions by

Eys and Peters (1981) based on infusion of very high concentrations of exogenous  $\alpha$ MSH do not corroborate our findings and we ascribe this to the concentrations used in that study. Probably, infusion demonstrates an effect, but not a real function of  $\alpha$ MSH as the concentrations used to reach this effect are non-physiologically high.

#### *Pigmentation – Effects of water acidification*

There were no differences in the pigmentation response to the different backgrounds between fish from neutral or low pH water during the pH decline over the first two days of the experiment. However, at the longer term the skin of the low water pH fish was lighter in pHG and pHB fish and slightly darker in pHW fish compared to fish kept at these backgrounds in neutral water. We take this to imply that the low water pH induced a mild stress that compromised the background adaptation process. On the other hand, acidification after 25 days for two days did only temporarily influence the pigmentation, since from day 27 to day 50 NpH fish restored their former pigmentation pattern and continued their background adaptation process (NpHB fish continued to darken and NpHW and NpHC fish continued to lighten). Apparently, when background adaptation is occurring under low pH water conditions, the background adaptation is slightly hampered. When fish have already adapted for 25 days to a given background, a decrease in water pH does no longer influence the pigmentation response. According to Ginneken et al. (1997) and Lamers et al. (1994), tilapia are able to handle a low pH quite well as long as the water pH is lowered gradually, for instance over a two-day period. The response of the tilapia in our study confirms that a two-day decrease of pH from 7.8 to 4.5 is slow enough not to disrupt hydromineral homeostasis.

#### *Plasma analysis – Effects of background adaptation*

Plasma cortisol and glucose showed a significant correlation with background colour. On a black background (NB), cortisol values were generally higher than plasma cortisol levels in NW fish. This indicates that a dark (black) background is experienced as stressful by tilapia. This has also been reported for rainbow trout (Green et al., 1991; Green and Baker, 1991). Black background adapted rainbow trout have higher cortisol levels than white background adapted fish, a difference that was increased by stressors (Green et al., 1991).

Interestingly, the increase in plasma cortisol in black background trout was accompanied by decreased MCH levels in plasma and in several brain regions (Green et al., 1991). MCH is known to inhibit the release of  $\alpha$ MSH from the pituitary gland in tilapia (Gröneveld et al., 1995). For the same species of fish as used in the present study, it was

reasoned that the decreased MCH levels and the accompanying loss of the inhibiting tonus on the release of  $\alpha$ MSH, may result in increased release of  $\alpha$ MSH from the pituitary gland during stress in tilapia, thereby evoking increased release of cortisol (Balm et al., 1995). Our present results are in agreement with this notion.

#### *Plasma analysis – Effects of water acidification*

Water acidification, either immediately (pH group) or after 25 days of undisturbed adaptation (NpH group), did not result in significant changes in plasma pH. Plasma pH values between 7.3 and 7.5 have been reported to be normal values in fish (Hirata et al., 2003), which correspond well with the values found in our study. A two-day decrease of water pH was not experienced as stressful in the present study as indicated by the lack of a significant rise in plasma pH and cortisol levels, which is in line with previous reports (Ginneken et al., 1997; Lamers et al., 1994), in which either a 6h or a 24h period of gradual water acidification was applied. However, plasma pH levels were generally lower when water acidification (water pH at 4.5) was applied after 25 days of undisturbed adaptation, indicating a reduced capacity to maintain plasma pH levels. Lamers and co-workers (1994) reported increased activity of melanotrope cells in the pituitary gland of tilapia exposed to a water pH of 4.5 for 7 days. In another study by these authors, plasma  $\alpha$ MSH levels were about 5-fold higher in low pH stressed fish (Lamers et al., 1992). In our study, a 3-fold increase was observed only in the NpH fish that had been left to adapt to the different backgrounds undisturbed in neutral water for 25 days before the water was acidified to pH 4.5. For NpHG fish, this increase was only temporary, while NpHB and NpHW fish continued to have elevated plasma  $\alpha$ MSH levels.

In the pituitary gland, the  $\alpha$ MSH content increased during the first two days of the experiment and during the two-day acidification after 25 days of background adaptation. The pituitary  $\alpha$ MSH content did not differ between fish from neutral water and fish from low pH water (both pH and NpH). This indicates that the increased plasma  $\alpha$ MSH levels during the final 25 days of the experiment in the NpH fish may have resulted from factors such as increased turnover of  $\alpha$ MSH in the pituitary gland or a decrease in  $\alpha$ MSH degradation in the plasma. Fish from the NpH group had lower lactate values (significant only in NpHG fish) and lower Na values (significant in NpHW fish) after acidification than fish from the pH groups. Plasma glucose was not affected, whereas cortisol levels increased strongest in NpH fish. Therefore, acidification starting at day 25, following a 25-day period of undisturbed background adaptation, induces a stronger rise in plasma  $\alpha$ MSH and cortisol levels and a

stronger decrease in plasma Na and lactate levels than acidification starting at day 0, simultaneously with the initiation of the process of background adaptation. This suggests that when fish have been left to adapt undisturbed to a certain background for 25 days, a subsequent acidification of the water has a higher stress-impact on the fish than water acidification immediately after transfer to the experimental tanks. We interpret this as an arousal of the stress axis in these fish.

These findings point to a reduction of the impact of either water acidification or background adaptation when they are both applied simultaneously. Such a reduced stress response to cope with multiple stressors has also been reported in other species of fish and in mammals (Ortuno et al., 2002; Sloman et al., 2002; Wasmund et al., 2002).

We conclude that when tilapia is subjected to different mild stressors simultaneously, this results in a less intense stress response than when exposure to a background or to water acidification is applied separately. This is also supported by our findings that adjustment of the hue to the respective backgrounds under low water pH conditions seems to be compromised compared to background adaptation in neutral water. Interestingly, while the stress response was more intense in the fish that could adapt without any disturbance to the different backgrounds for 25 days prior to the decrease in water pH, these fish showed a recovery of the hue of the skin shortly after the pH decrease and during the remainder of the experiment. This indicates once more that when fish have already accomplished a new homeostatic equilibrium, a subsequent stressor will not interfere with the previous adaptation process. The two adaptation processes studied here apparently do show interference when occurring simultaneously, reducing the individual impact of the stressors applied.

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## Legends to the figures

**Figure 1 – (A)** Darkness of the scales as expressed in % of the total area of scale that is covered by melanophores (+ sd). Fish were kept on three different backgrounds (translucent glass resulting in a grey background, ▲; black, ■; and white, ●) undergoing different water acidification treatments (no acidification, N, closed symbols; direct acidification, pH, open symbols; and acidification after 25 days in neutral water, NpH, closed symbols with dashed line). Significant differences ( $P < 0.05$ ) from time 0 control (indicated by the dotted line in all graphs) are indicated by \*; differences between corresponding values from different backgrounds are indicated by letters; a vs. b =  $P < 0.05$ . **(B)** Tilapia kept in neutral water for 25 days with corresponding scale; upper: black fish, with a scale melanophore coverage of 36.23% and lower: white fish with a scale melanophore coverage of 5.39%

**Figure 2 – Plasma cortisol (A),  $\alpha$ MSH (B) and  $\alpha$ MSH content of the pituitary gland (C)** for fish kept on three different backgrounds (translucent glass resulting in a grey background, ▲; black, ■; and white, ●) undergoing different water acidification treatments (no acidification, N, closed symbols; direct acidification, pH, open symbols; and acidification after 25 days in neutral water, NpH, closed symbols with dashed line). Significant differences are indicated by \* = different from t 0 control, or different letters;  $P < 0.05$  in both cases.

**Table 1 - Experimental set-up.** Fish were kept on three different backgrounds (translucent glass as a reference control; black and white) and allowed to adapt in either neutral water for 25 days (N), followed by a pH drop (7.8 to 4.5) over a two-days period after which the pH was kept at 4.5 (NpH); or a pH drop and subsequent low pH immediately after the start of the experiment (pH).

**Table 2 - Plasma pH, glucose, lactate and Na values** for fish kept on a translucent control (C), black (B) or white (W) background. Fish were acidified (water pH from 7.8 to 4.5) either immediately after the start of the experiment (pH group) or first allowed to adapt to the different backgrounds in neutral water (N) before the water was acidified (NpH). The values of these two groups are depicted underneath one another. Values of

fish from low pH water are boxed in grey. Significant differences between values are indicated with letters; a vs. b =  $P < 0.05$ , a vs. c =  $P < 0.01$ ; values significantly different from day 0 initial values are indicated with \* ( $P < 0.05$ ).

**Table 3** – Correlation table between experimental factors and physiological parameters. A positive correlation is indicated by + and a negative correlation is indicated by -. Significant correlations are indicated by \* for a correlation significant with  $P < 0.05$  and \*\* for  $P < 0.01$ . Non-significant correlations are not shown.

Table 1

	Background:	G	B	W
Water treatment:	Days of treatment:			
N	0-25	NG	NB	NW
pH	0-25	pHG	pHB	pHW
NpH	25-50	NpHG	NpHB	NpHW

Table 2

day	pH		glucose		lactate		Na	
	NG; NpHG	pHG	NG; NpHG	pHG	NG; NpHG	pHG	NG; NpHG	pHG
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.25±0.09	7.26±0.08	3.6±0.4	3.9±1.0	1.4±0.3 <sup>a</sup>	1.5±0.4 <sup>a</sup>	173±3	174±4
8	7.21±0.17	7.34±0.09	4.0±0.4 <sup>a</sup>	3.9±0.4	1.8±0.6 <sup>a</sup>	1.4±0.4 <sup>a</sup>	173±4	170±7 <sup>b</sup>
25	7.28±0.09	7.22±0.17	3.5±0.2 <sup>b</sup>	3.8±0.7	1.2±0.4	1.2±0.5	176±6	179±7
27	7.24±0.13		3.6±0.3		1.4±0.5		167±5	
33	7.25±0.06		n.a.		0.8±0.1 <sup>b*</sup>		163±2	
50	7.16±0.22		4.0±0.4		0.8±0.1 <sup>b*</sup>		169±5	
	NB; NpHB	pHB	NB; NpHB	pHB	NB; NpHB	pHB	NB; NpHB	pHB
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.30±0.05	7.26±0.05	3.6±0.6	4.0±0.8	1.3±0.2	1.4±0.3	171±4	173±3
8	7.29±0.08	7.28±0.08	3.8±0.9	3.5±0.4	1.1±0.4	1.3±0.4	167±4 <sup>b</sup>	178±4 <sup>a</sup>
25	7.30±0.10	7.24±0.13	3.2±0.2	3.3±0.4	1.1±0.3	1.4±0.5	174±6	175±5
27	7.24±0.13		4.0±0.6		1.1±0.1 <sup>*</sup>		174±11	
33	7.27±0.07		3.7±0.2		1.1±0.4		168±3 <sup>b</sup>	
50	7.17±0.12		3.6±0.3		1.2±0.5		167±5	
	NW; NpHW	pHW	NW; NpHW	pHW	NW; NpHW	pHW	NW; NpHW	pHW
0	7.26±0.02		3.5±0.8		1.5±0.1		170±2	
2	7.29±0.06	7.25±0.08	3.4±0.5	5.0±1.8	1.2±0.3	1.4±0.3	172±4 <sup>b</sup>	175±4 <sup>ab</sup>
8	7.33±0.07	7.26±0.08	3.6±0.4	3.7±0.3	1.6±0.5	1.3±0.3	172±5 <sup>bc</sup>	180±2 <sup>a*</sup>
25	7.23±0.09	7.28±0.09	3.4±0.3	3.6±0.2	1.2±0.4	1.6±0.4	176±6 <sup>ab</sup>	177±6 <sup>ab</sup>
27	7.23±0.10		4.0±0.2		1.1±0.3		165±3 <sup>c</sup>	
33	7.25±0.09		4.5±0.6		1.5±0.3 <sup>a</sup>		173±3 <sup>b</sup>	
50	7.18±0.15		3.8±0.3		1.0±0.3		165±2 <sup>c</sup>	

Table 3

	<b>pH 7.8 ⇒ 4.5</b>	<b>B⇒G ⇒W</b>	<b>Cortisol</b>	<b>αMSH</b>	<b>αMSH pituitary</b>	<b>Glucose</b>	<b>Na</b>	<b>Scale coverage</b>
<b>pH 7.8 ⇒ 4.5</b>	---			+ 0.253**		+ 0.251**	- 0.137*	- 0.309**
<b>B⇒G ⇒W</b>		---	- 0.152**			+ 0.170*		- 0.551**
<b>Cortisol</b>		- 0.152**	---		+ 0.444**			
<b>αMSH</b>	+ 0.253**			---	- 0.228*		- 0.157**	
<b>αMSH pituitary</b>			+ 0.444**	- 0.228*	---	+ 0.241*		
<b>Glucose</b>	+ 0.251**	+ 0.170*			+ 0.241*	---		
<b>Na</b>	- 0.137*			- 0.157**			---	
<b>Scale coverage</b>	- 0.309**	- 0.551**						---



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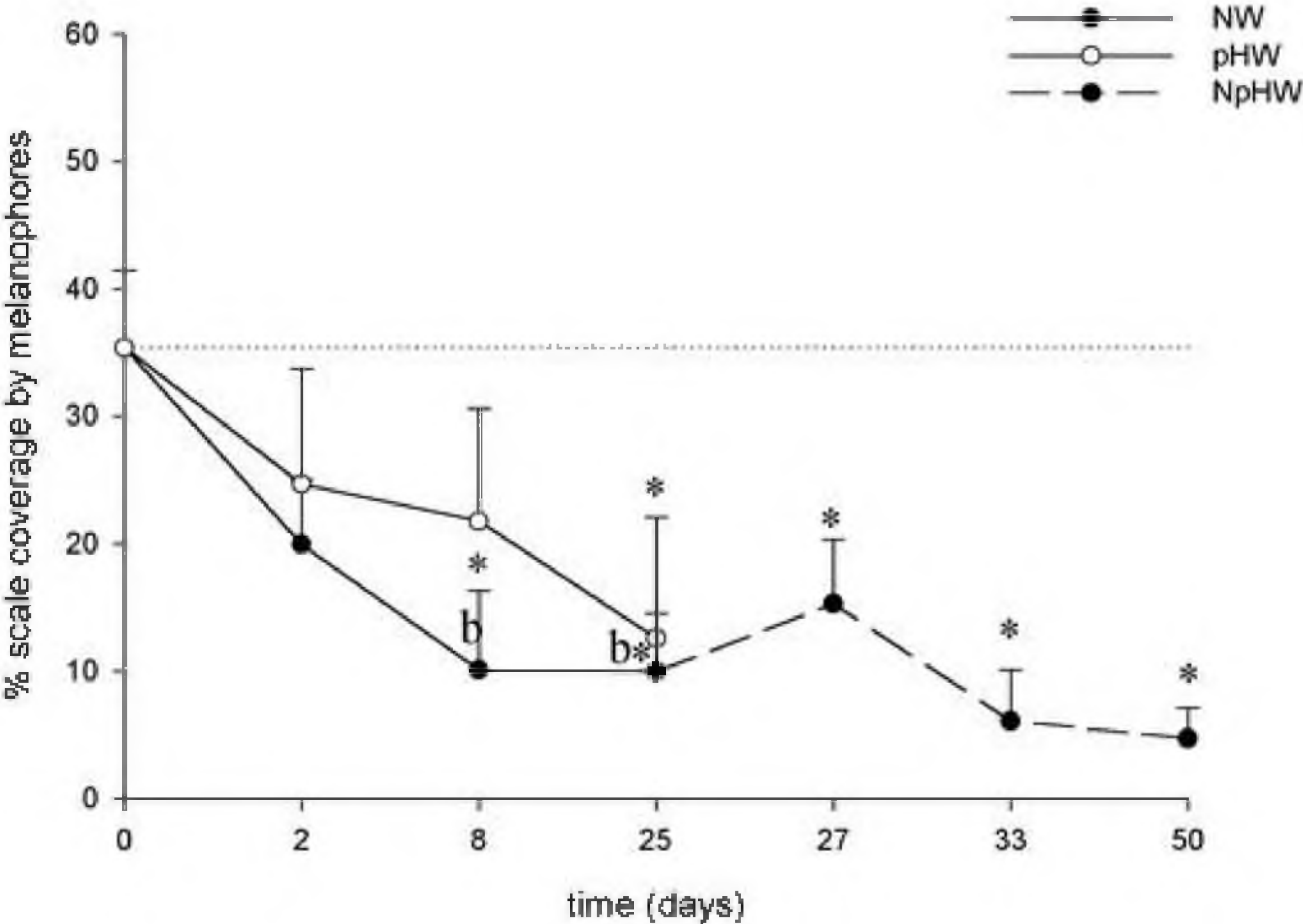


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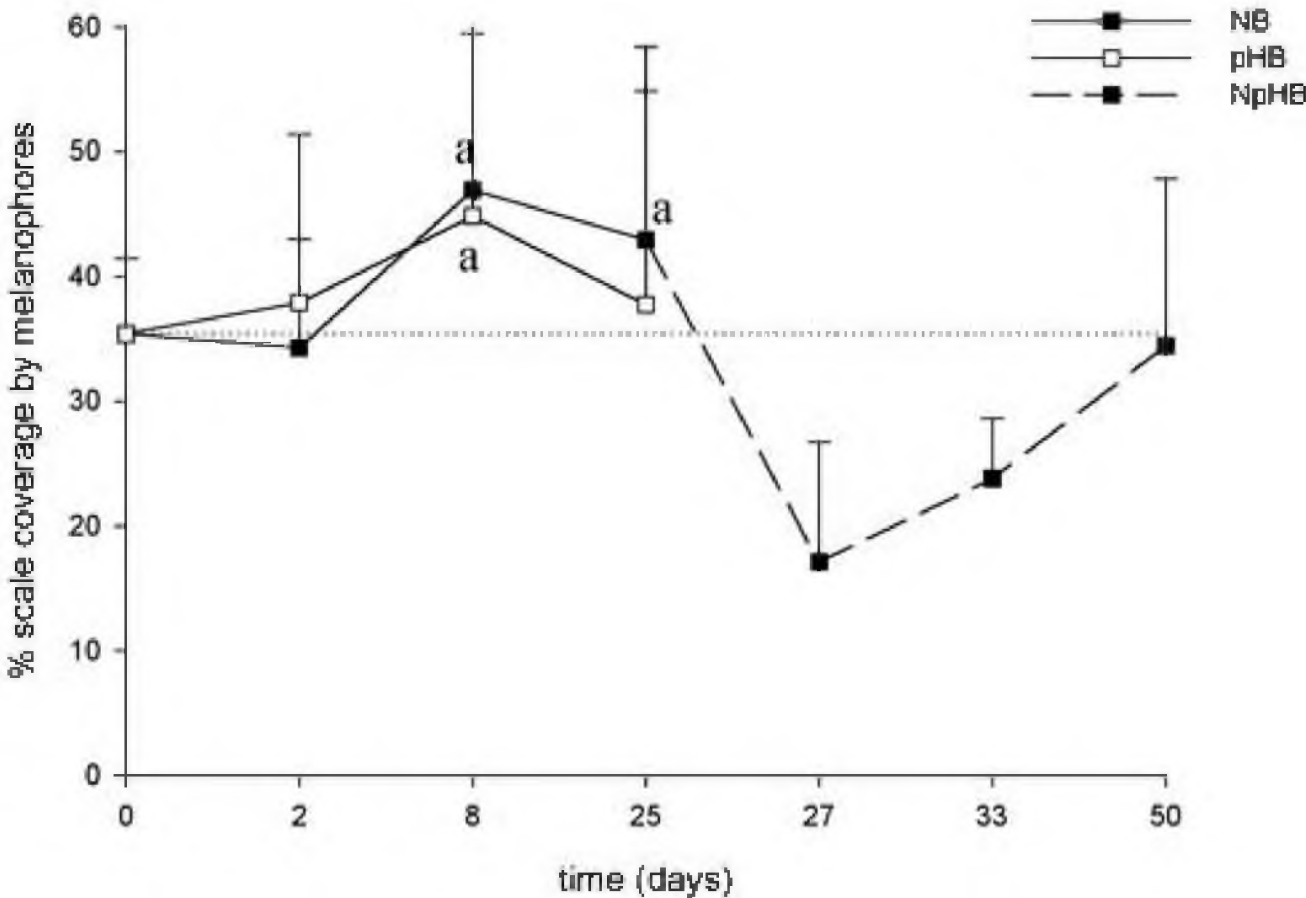
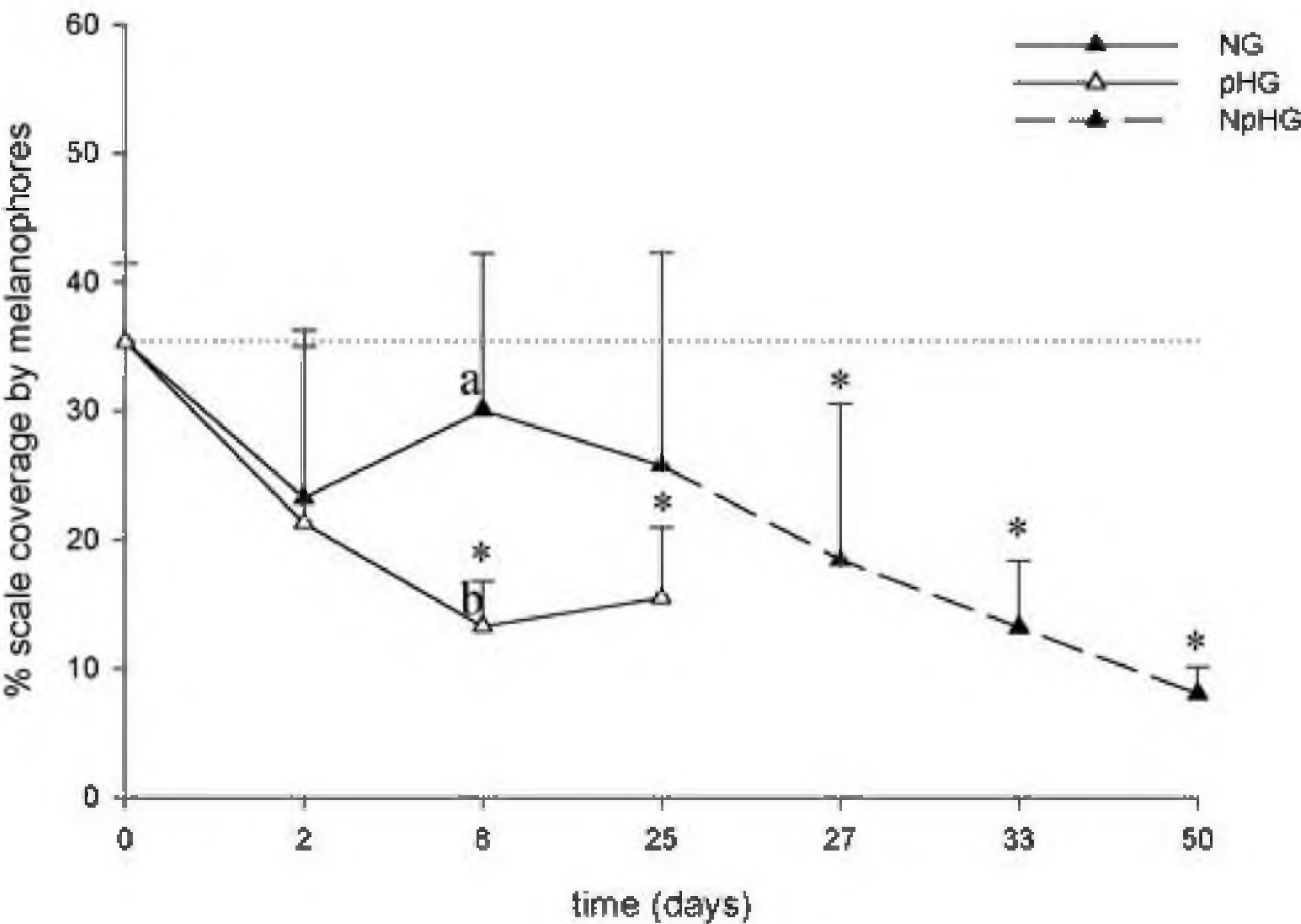


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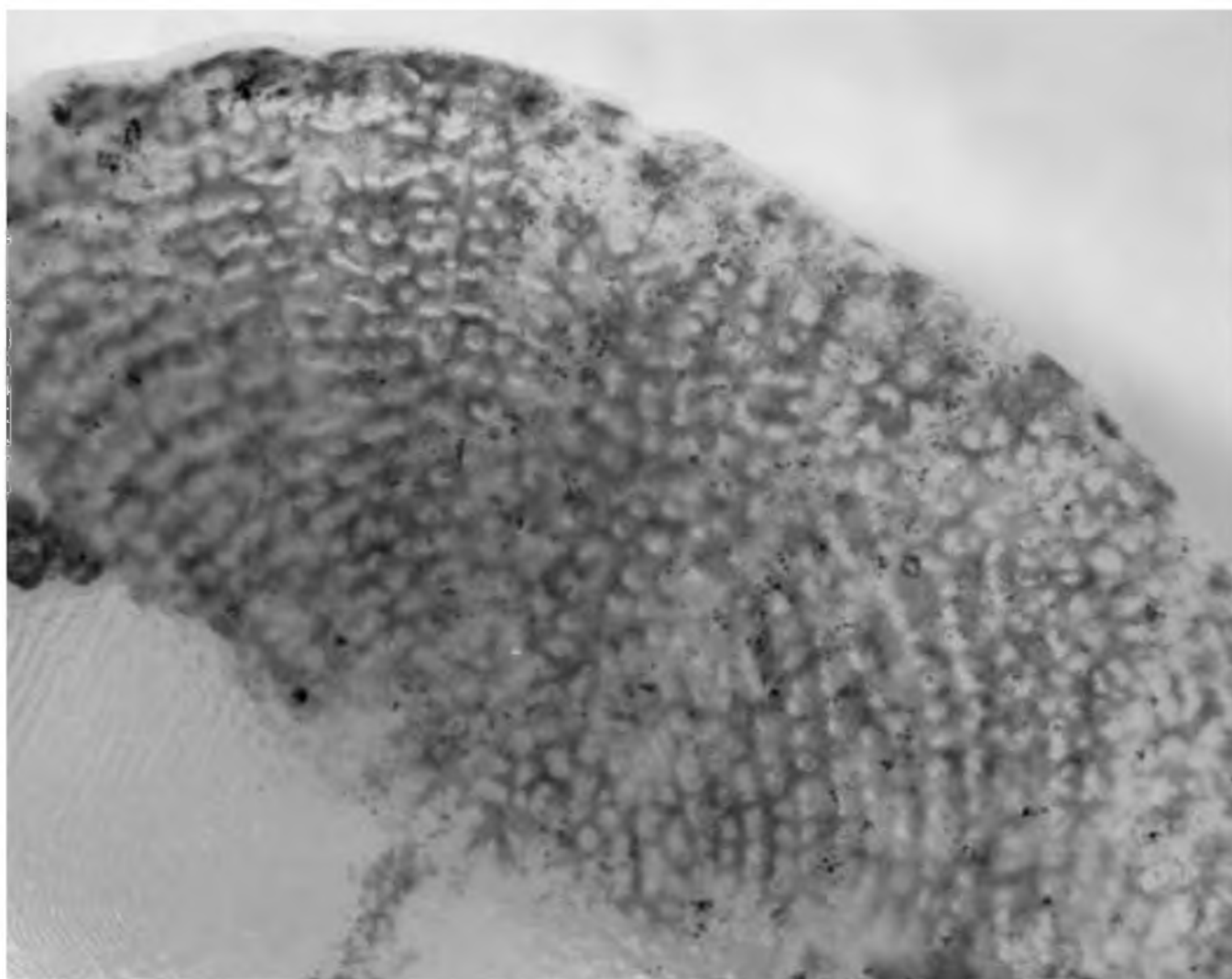


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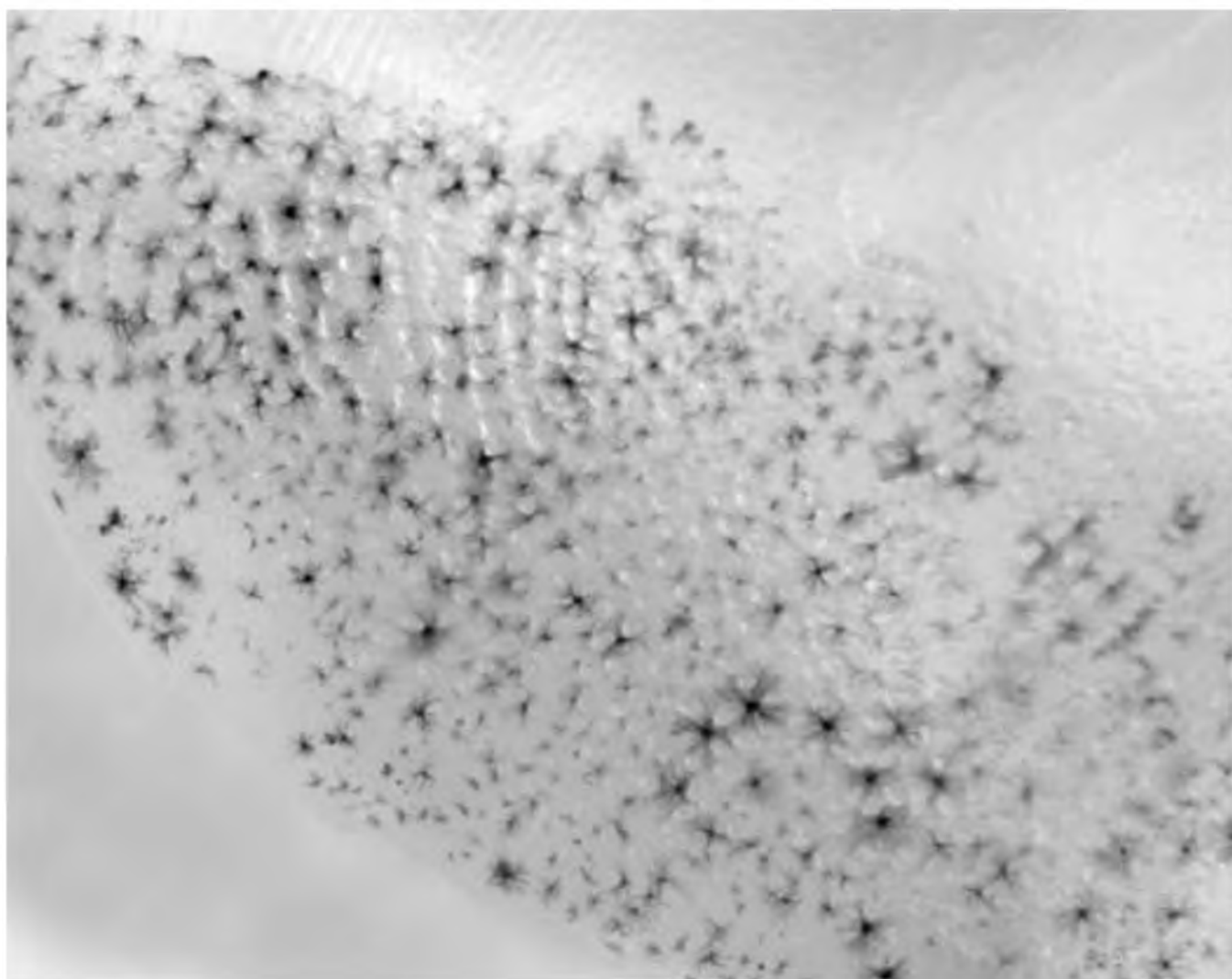


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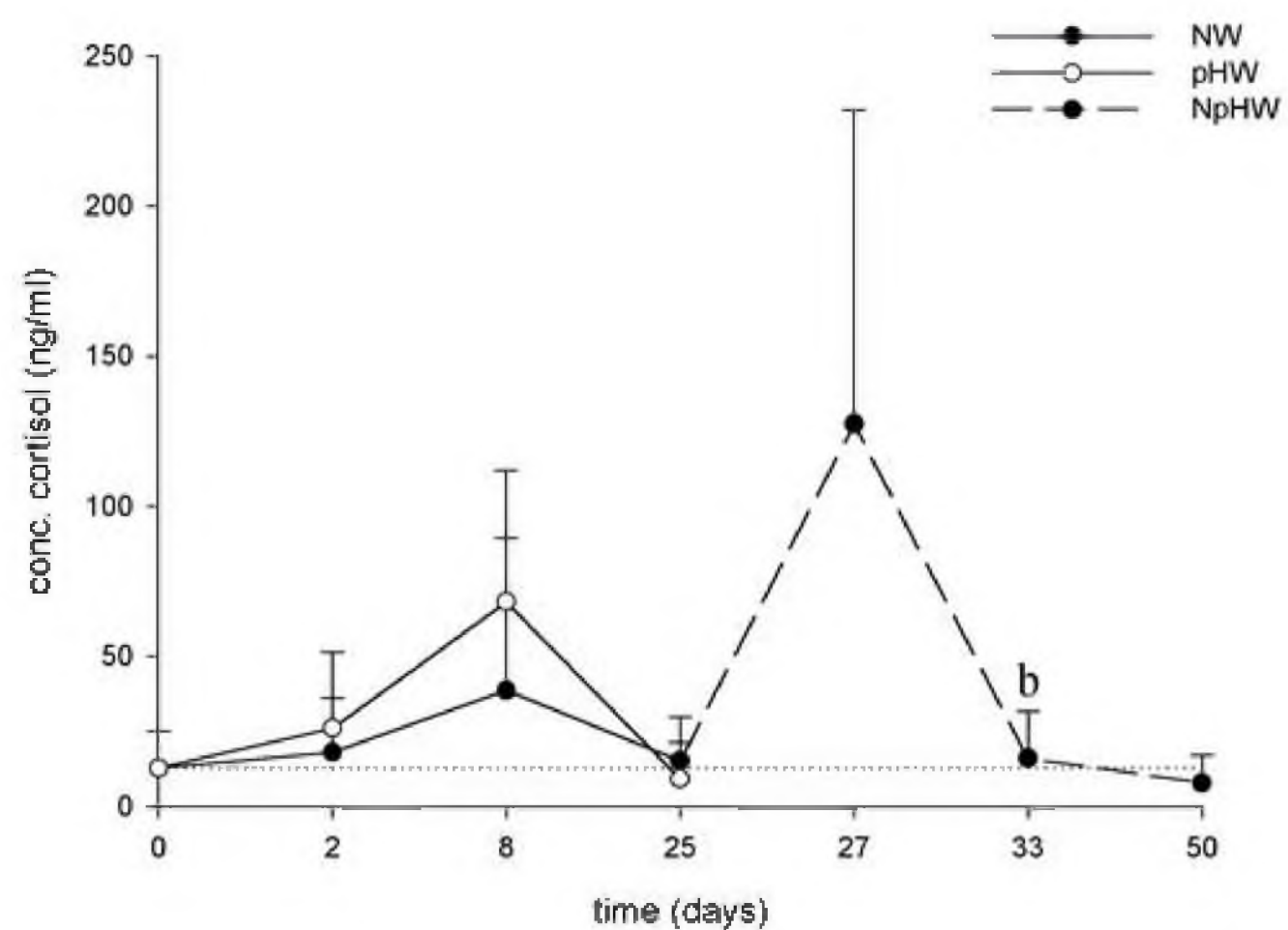




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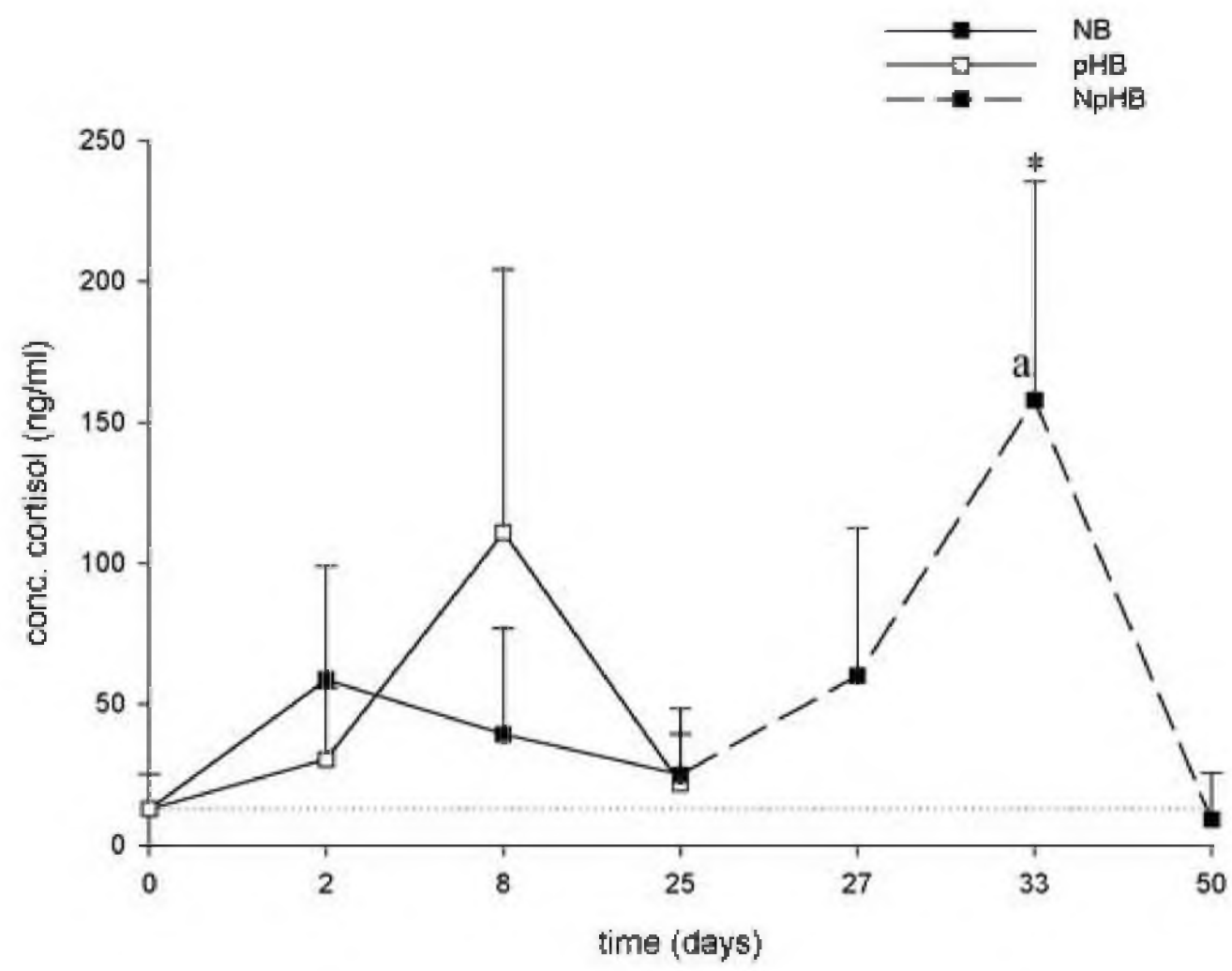


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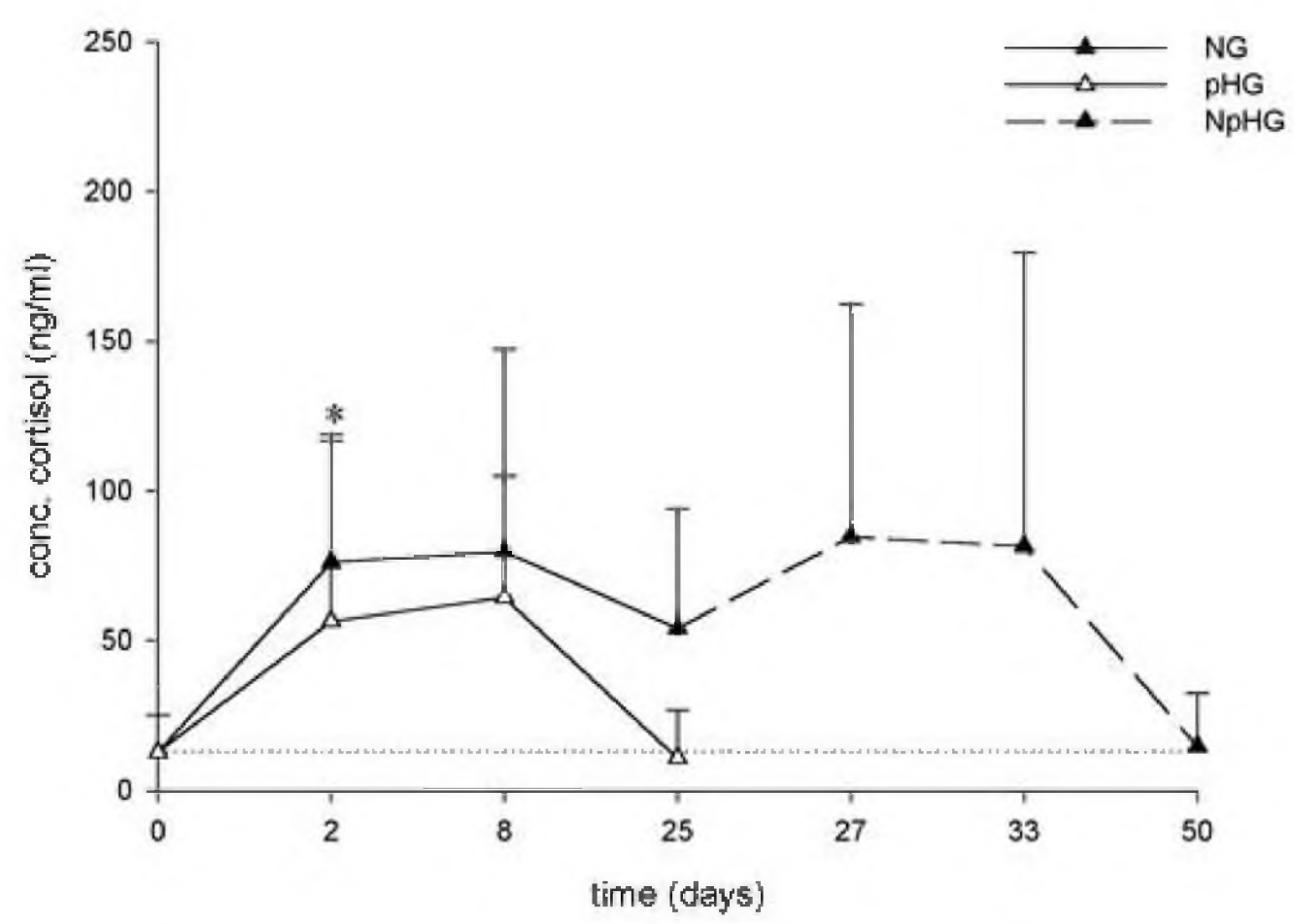


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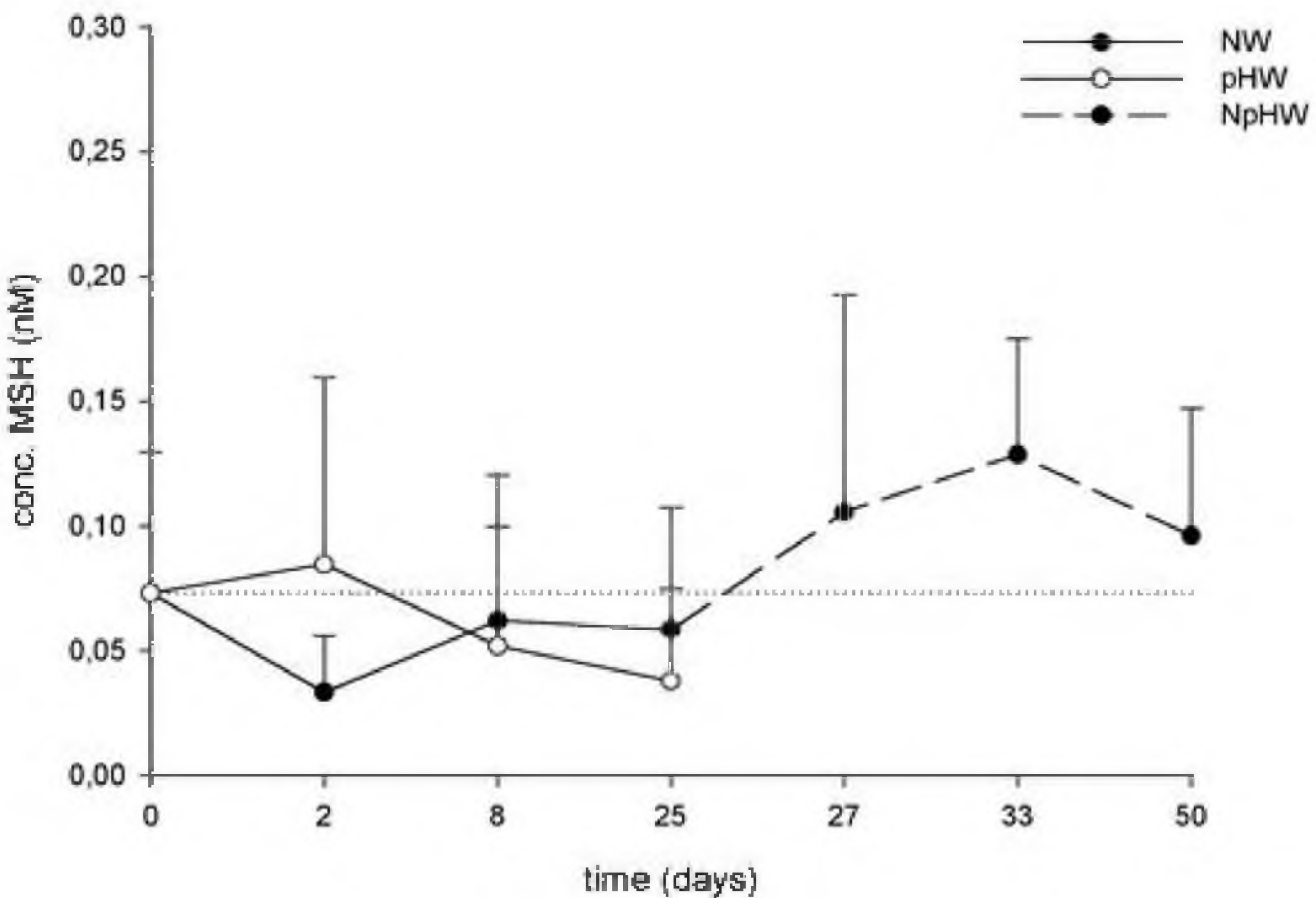


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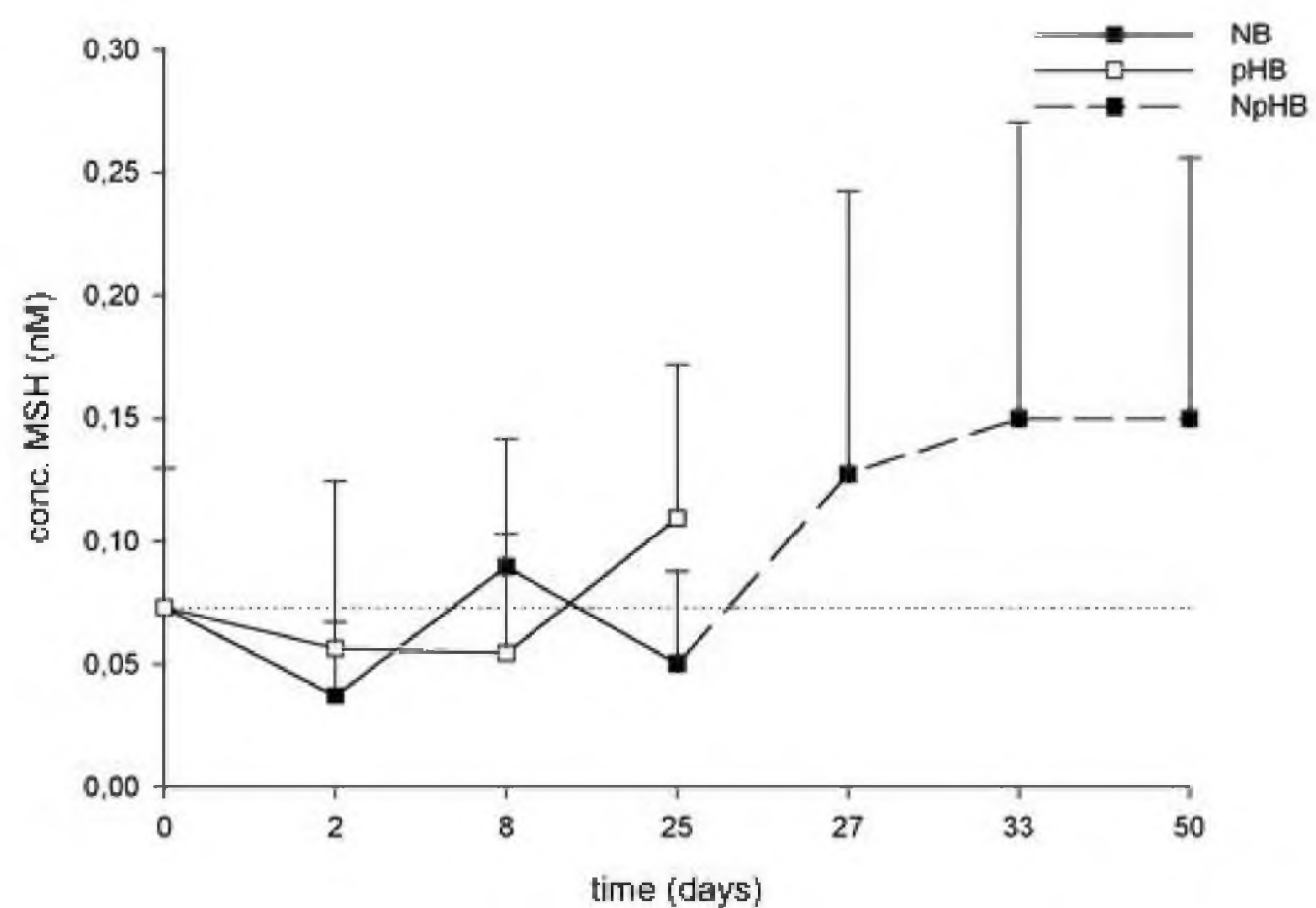


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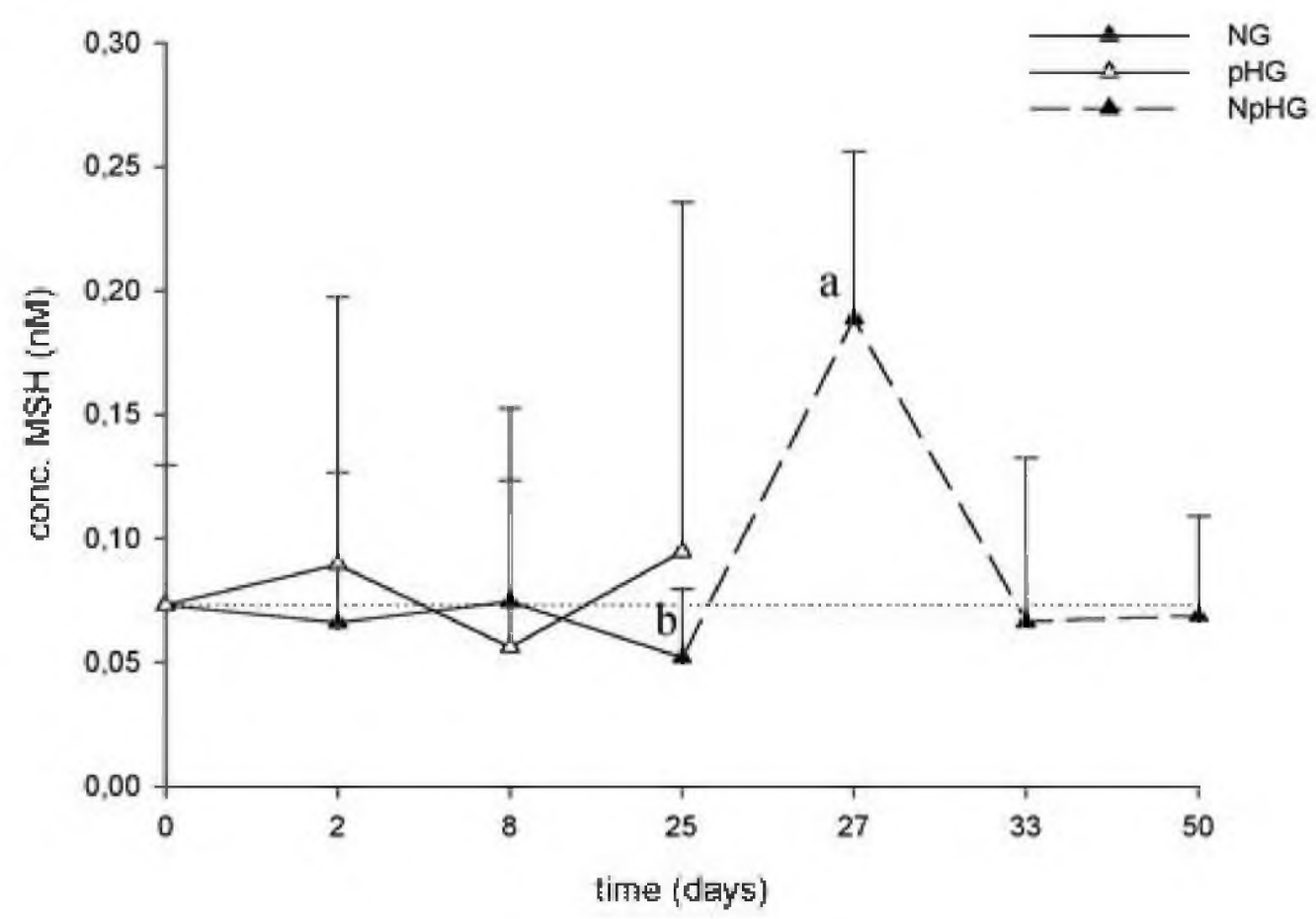


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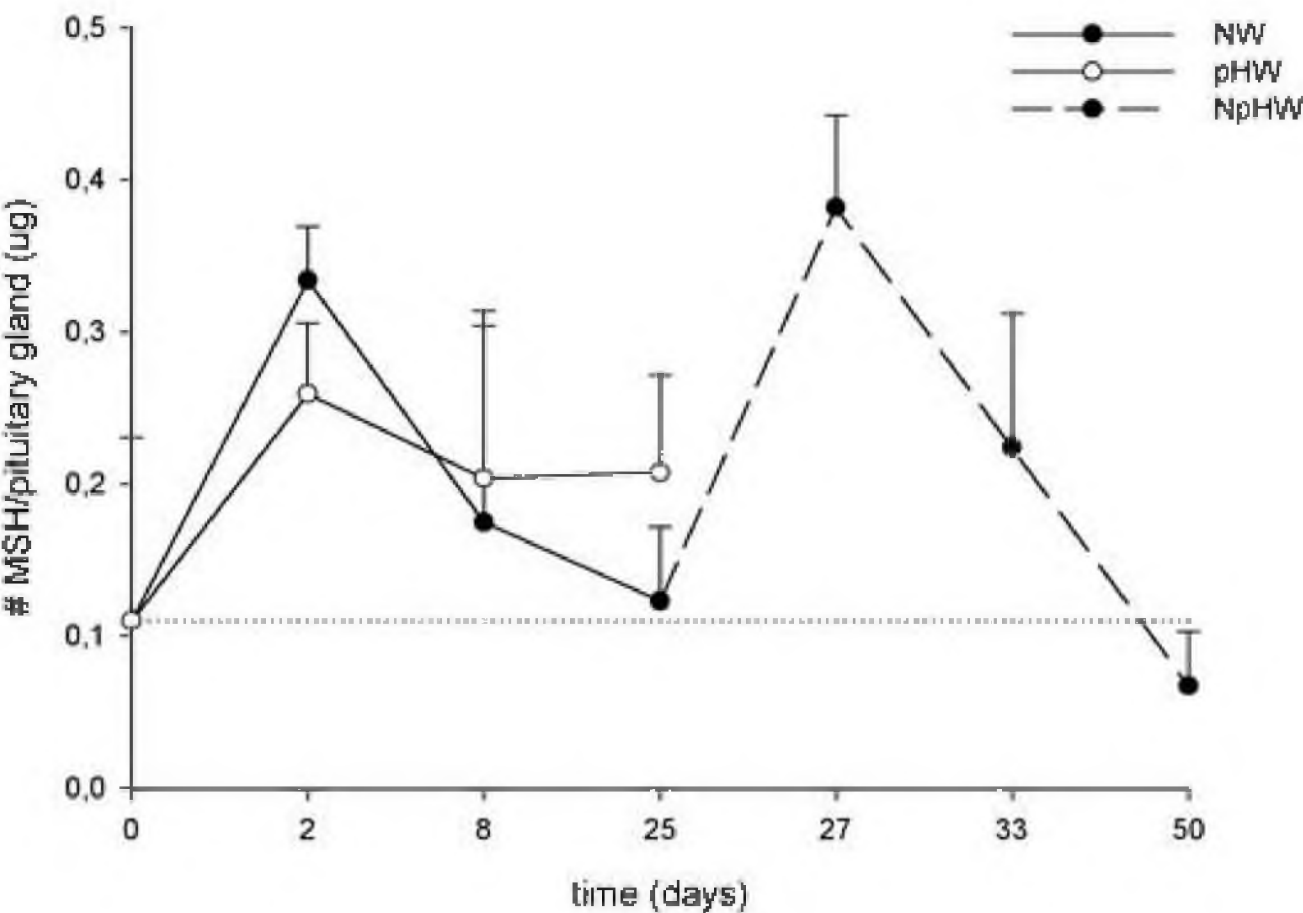


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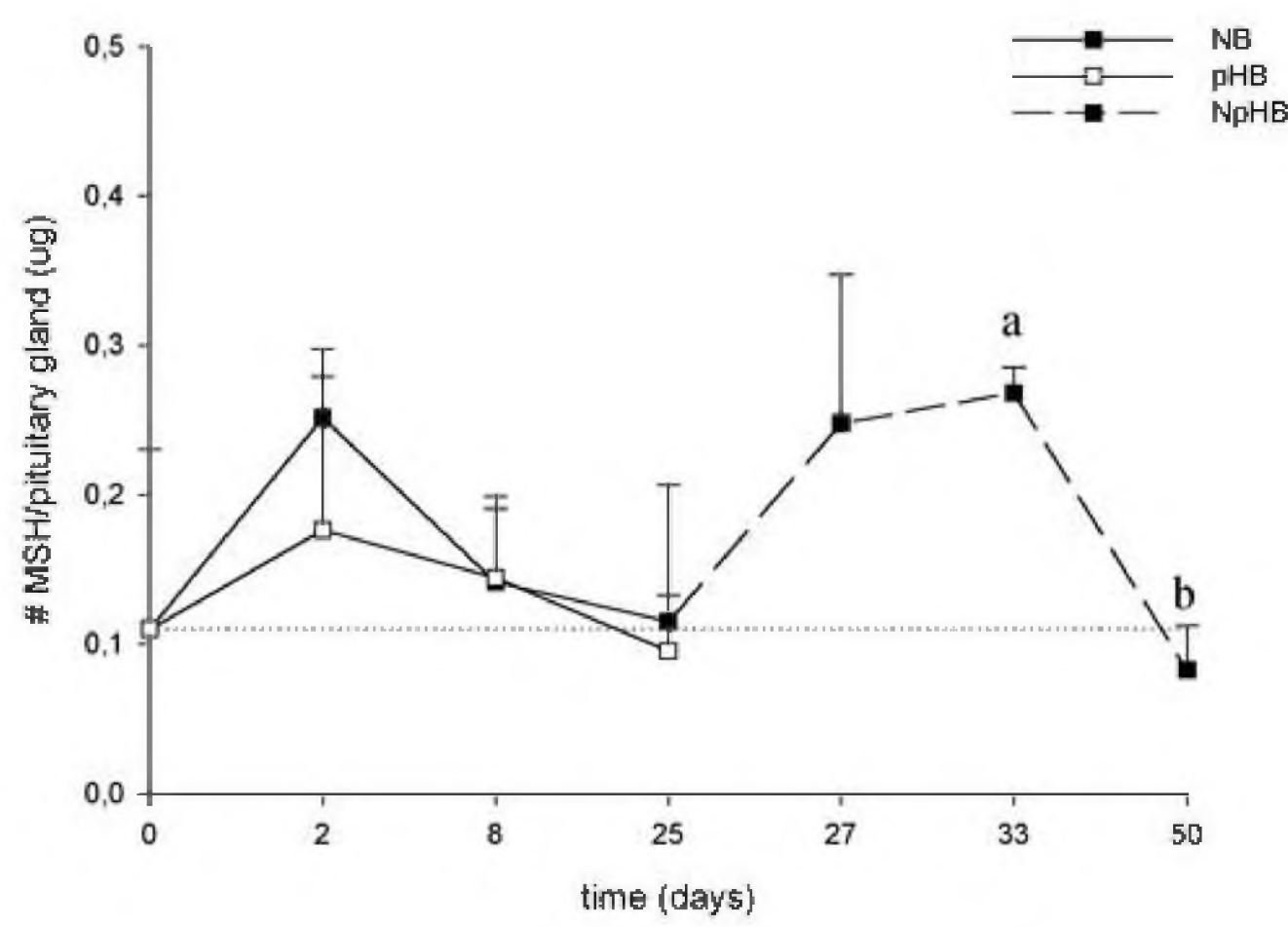


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